Title

A comparison of methods for determining ploidy in white sturgeon (Acipenser transmontanus)

Authors

Fiske, Julia A
Goodbla, Alisha M
Holem-Bell, Cara E
et al.

Publication Date

2019-05-01

DOI

10.1016/j.aquaculture.2019.03.009

Peer reviewed

Aquaculture 507 (2019) 435–442

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture

A comparison of methods for determining ploidy in white sturgeon (*Acipenser transmontanus*)

Julia A. Fiske[a,*], Joel P. Van Eenennaam[a], Anne E. Todgham[a], Shawn P. Young[b], Cara E. Holem-Bell[b], Alisha M. Goodbla[a], Andrea D. Schreier[a]

[a] Department of Animal Science, University of California, 2235 Meyer Hall, 1 Shields Ave, Davis, CA, United States
[b] Kootenai Tribe of Idaho, 242 Hatchery Road, Bonners Ferry, ID, United States

ARTICLE INFO

Keywords:
Ploidy
Autopolyploid
Acipenser transmontanus
Broodstock
Erythrocytes

ABSTRACT

Release of sturgeon with abnormal ploidy into the wild may result in reduced fitness due to lowered fertility in the F_2 and subsequent generations. Further, there is evidence that ploidy affects reproductive development and caviar yield. Therefore, the ability to accurately characterize the ploidy of white sturgeon (*Acipenser transmontanus*) is essential for both commercial and conservation aquaculture. This study compares nuclear volume and whole erythrocyte long-axis lengths obtained using Coulter counter and blood smears, respectively, from captive white sturgeon from populations originating in California and Idaho. We examine which method provides the most accurate, time efficient and cost-effective characterization of ploidy in this species. Results from Coulter counter and blood smears were compared to results from flow cytometry, the gold standard for genome size analysis. Previous work suggests that blood smears can distinguish between 8N (diploid) and 12N (triploid) sturgeon, but further analysis is required to see if this method can also be used to identify 10N fish and to provide robust evidence of its utility in 8N and 12N fish across populations. In this study, we demonstrated that the Coulter counter had 100% agreement with flow cytometry in ploidy assignment, while blood smears vary in their accuracy based on population. Blood smears showed a high degree of overlap in erythrocyte long-axis length between 8N and 10N individuals as well as some overlap between 10N and 12N individuals in the California fish, and a high degree of overlap between 8N and 12N individuals in the Idaho fish. Although blood smears are time-intensive and vary in their ploidy assignment accuracy, they are a low-cost technique and as such may have some utility for caviar farms attempting to identify 12N individuals in a small number of broodstock. By comparing the accuracy, efficiency and cost of these three methods, sturgeon farmers and conservation hatcheries will be able to choose the best method for their needs in determining the ploidy of their fish. We determined that Coulter counter is equally accurate to flow cytometry and is also the most time efficient method for ploidy determination in white sturgeon.

1. Introduction

White sturgeon (*Acipenser transmontanus*) are a highly prized aquaculture species for both their meat and caviar, and wild populations are of conservation concern due to habitat destruction and overfishing (Duke et al., 1999; Hildebrand et al., 2016). Modern sturgeon derive from an ancient lineage, and their evolution included multiple polyploidization events (Birstein et al., 1997). Normal white sturgeon are evolutionary octoploids (8N) with ~240 chromosomes but in culture, white sturgeon can exhibit spontaneous autopolyploidy, the duplication of the maternal genome due to retention of the second polar body, resulting in the creation of 12N individuals with ~360 chromosomes (Schreier et al., 2013). Thus far, these genome duplications have not been intentionally induced by aquaculturists, but preliminary evidence suggests that a combination of egg quality in individual females, post-ovulatory egg ageing, and mechanical shock during the egg deadhesion process may contribute to spontaneous autopolyploidy (Schreier and Van Eenennaam, unpublished data). Both 8N and 12N individuals are fertile and mating between 8N and 12N individuals results in offspring of intermediate ploidy (10N) with ~300 chromosomes (Hedrick et al., 1991; Birstein, 2005; Schreier et al., 2011). There may be some positive effects of larger genome size, such as possible larger egg size from 12N females, a desirable quality for caviar. However, use of 12N individuals as broodstock for mating with

* Corresponding author.
E-mail address: afiske@ucdavis.edu (J.A. Fiske).

https://doi.org/10.1016/j.aquaculture.2019.03.009
Received 12 October 2018; Received in revised form 4 February 2019; Accepted 8 March 2019
Available online 09 March 2019
0044-8486/

8N individuals may result in 10N progeny. Although 10N males mature normally, the females exhibit delayed maturation and therefore increased time to harvest for caviar (Schreier and Van Eenennaam, 2018). Similar results of matings between individuals with differing ploidy levels has been observed in Siberian sturgeon (*Acipenser baerii*) (Havelka et al., 2014, 2016). Furthermore, spontaneous autopolyploids are poor candidates for release into the wild from conservation hatcheries, which sustain several white sturgeon populations throughout North America, due to negative effects such as delayed reproductive maturation in the F_2 and subsequent generations (Schreier and Van Eenennaam, 2018). For these reasons, ploidy is an important characteristic for commercial farmers to consider when selecting individuals for broodstock, and for conservation hatcheries to consider when releasing fish. Thus, the ability to accurately identify the ploidy of an individual while minimizing cost is an important step in actively managing sturgeon in culture.

Erythrocyte major and minor axes lengths obtained from blood smears have been demonstrated to correspond to ploidy in a variety of other fish species, including Atlantic salmon (*Salmo salar*) (Cogswell et al., 2002), saugeyes (female walleye *Sander vitreus* and male sauger *Sander canadensis*) (Garcia-Abiado et al., 1999), and blue tilapia (*Tilapia aurea*) (Valenti, 1975) with fish of higher ploidy showing larger erythrocyte size (Sezaki et al., 1977; Lemoine and Smith, 1980). Among sturgeon, 8N and 12N shortnose sturgeon (*Acipenser brevirostrum*) exhibited significant differences in erythrocyte long-axis length (Beyea et al., 2005). More traditional methods of ploidy evaluation, including flow cytometry (Thorgaard et al., 1982; Allen, 1983; Johnson et al., 1984; Harrell et al., 1998) and Coulter counter analysis (Johnson et al., 1984; Wattendorf, 1986; Van Eenennaam et al., 1996; Harrell et al., 1998), require expensive equipment that may be cost prohibitive for many small caviar farms and conservation hatcheries. Flow cytometry analysis is considered the gold standard method of ploidy determination to which Coulter counter and blood smear analysis are compared in this study. Flow cytometry calculates mean nuclear DNA content by measuring the fluorescence emitted by stained nuclear DNA (Allen, 1983). The level of fluorescence is compared to a known standard, with 10,000 cells measured per sample. In Coulter counter analysis, cells are suspended in an electrolyte solution, and cause a drop in electrical current as they pass through the instrument's aperture. The drop in electrical current is proportional to particle size, which is used as a proxy for nuclear DNA content, with approximately 20,000 cells measured per sample (Wattendorf, 1986). Using blood smears to determine ploidy is a relatively inexpensive technique, and thus holds promise for reducing costs for farmers and conservation hatcheries alike. Previous work in our laboratory suggests that 8N and 12N white sturgeon can be differentiated based on the long-axis length and area of erythrocytes (Schreier and Gille, unpublished data); however, preliminary work only examined 8N and 12N fish from one family and one population. Further work is required to look at variation in these measures among populations and families to evaluate the accuracy and repeatability of using blood smears to differentiate 8N, 10N, and 12N individuals. In this study, we aim to compare ploidy class assignments obtained by flow cytometry, Coulter counter and blood smears in a set of 238 white sturgeon from two populations containing 8N, 10N and 12N individuals to compare accuracy and efficiency across methods.

2. Materials and methods

2.1. Sample origin

White sturgeon used in this study originated from either Sterling Caviar LLC in Elverta, CA (a population established from the Sacramento River and hereafter termed California fish), or the Kootenai Tribe of Idaho Native Fish Conservation Aquaculture Program (established from the Kootenai River and hereafter termed Idaho fish). California fish were progeny obtained from a prior study of how post-ovulatory ageing affects the incidence of spontaneous autopolyploidy. Milt from two 8N males was used to fertilize ovulated eggs from two 8N females, one pair in 2015 and one pair in 2016. A group of twenty-nine 8N progeny was selected from the family created in 2015, and thirty each of 8N and 12N progeny were selected from the family created in 2016. Intermediate ploidy (10N) progeny were created by fertilizing ovulated eggs from a 12N female with milt from an 8N male in 2015, and twenty-nine progeny were selected for this study (Table 1). Ploidy of all parents was determined using flow cytometry (see Flow cytometry below). All embryos were transported to the Center for Aquatic Biology and Aquaculture at the University of California, Davis for hatching and rearing. Spawning and rearing procedures followed methods described by Van Eenennaam et al. (2004), and followed the protocol approved by the UC Davis Institutional Animal Care and Use Committee (protocol #19778). At approximately 1 year of age, all fish were non-lethally bled for preliminary ploidy assignment using the Coulter counter and fish were separated by putative ploidy groups for continued rearing.

Table 1
Number of individuals belonging to each ploidy class (validated by flow cytometry) from the California fish (CA) and Idaho fish (ID).

Family	Year	8N	10N	12N
CA 1	2015	29	29	N/A
CA 2	2016	30	N/A	30
ID 1	2016	3	N/A	27
ID 2	2016	30	N/A	N/A
ID 3	2016	25	N/A	5
ID 4	2016	N/A	N/A	15
ID 5	2016	7	N/A	8
Total		124	29	85

Idaho fish were spawned and reared according to the Kootenai River White Sturgeon and Burbot Aquaculture Techniques Manual (Young et al., 2016). Five of the twenty-nine families produced in the 2016 year class were selected for this study. Thirty individuals of unknown ploidy were selected from families one, two and three, and fifteen individuals of unknown ploidy were selected from families four and five (Table 1).

2.2. Blood collection

Blood collection from the California fish took place during two separate sampling events; the first with twenty-nine putative 8N and 10N individuals at approximately 17 months of age (mean fork length = 62.2 cm, SE = 0.8 cm, mean weight = 1921 g, SE = 82 g), and the second with thirty putative 8N and 12N individuals at approximately 11 months of age (mean fork length = 53.2 cm, SE = 0.6 cm, mean weight = 1276 g, SE = 44.8 g). Five to six fish at a time were euthanized in a large ice chest containing 500 mg/L MS-222 buffered with 250 mg/L sodium bicarbonate. Five-hundred μL of whole blood was collected from each fish via puncture of the caudal vasculature with a 1 cc insulin syringe prepped with 500 μL acid citrate dextrose solution as an anticoagulant (ACD: 0.48 g citric acid, 1.32 g trisodium citrate, 1.47 g dextrose, H_20 to 100 ml) and attached to a 25 gauge needle (Blacklidge and Bidwell, 1993). After inverting several times, 3 μL of blood-ACD solution was transferred into a Coulter counter cuvette, 3 μL was placed on each of two microscope slides for blood smears, and the remainder (approximately 990 μL) was put into a FACS tube for flow cytometry.

Blood samples from the Idaho fish were obtained during three different sampling events in January (15.4 ± 1.8 cm, 21.9 ± 1.8 g, $n = 60$) February (15.5 ± 3.4 cm, 24.5 ± 1.6 g, $n = 30$) and March 2017 (19.4 ± 3.9 cm, 26.4 ± 1.7 g, n = 30), with ages of fish ranging from 10 to 12 months. Fish were anesthetized in a solution of 100 mg/L of MS-222 (tricaine methanesulfonate, Western Chemical, Inc. Ferndale, WA) and 213 mg/L of sodium bicarbonate and the entire solution was changed between every 30 fish sampled. Syringes were

Table 2
Results of Shapiro-Wilk normality tests.

Method type	Population	8N	10N	12N	Test used
Blood smears	CA	p = .7755 W = 0.98688	p = .8468 W = 0.98	p = .3932 W = 0.96413	One-way ANOVA
	ID	p = .029* W = 0.96	N/A N/A	p = 9.558e-05* W = 0.88771	Mann-Whitney U Test
Coulter counter	CA	p = 3.71e-07* W = 0.81425	p = 6.415e-08* W = 0.58281	p = 1.088e-07* W = 0.61404	Kruskal-Wallis Test
	ID	p = .0002811* W = 0.87766	N/A N/A	p = 1.088e-07* W = 0.61404	Mann-Whitney U Test

Asterisks denote results from non-parametric datasets. N/A refers to ploidy classes not analyzed in this study. Test used refers to test type used in subsequent analysis.

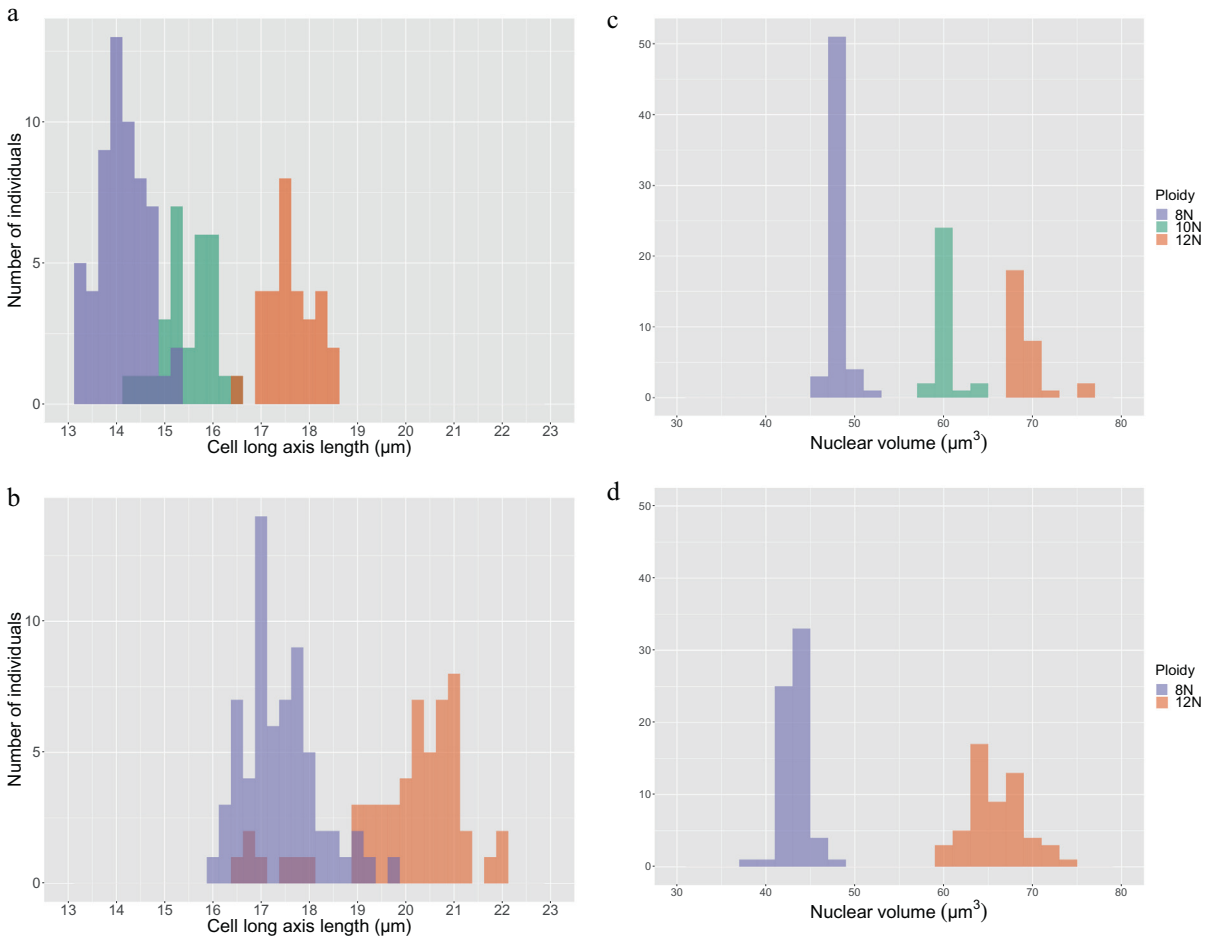

Fig. 1. Distribution of mean erythrocyte long-axis lengths for 8N (purple), 10N (green, California only) and 12N (orange) white sturgeon from the (a) California fish (n = 118) and (b) Idaho fish (n = 120) measured using blood smears. Distribution of modal nuclear volume for the (c) California fish (n = 118) and (d) Idaho fish (n = 120) measured using Coulter counter. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

prepped with 400–500 μL ACD and 400–500 μL of whole blood was collected via caudal venipuncture using a 23 gauge needle and 1 cc insulin syringe. After inverting the syringe containing the blood and ACD buffer several times, the mixture was transferred to FACS tubes containing 1 mL of ACD. The tubes were placed in racks and allowed to settle at 4 °C overnight. The following morning, tubes were placed in a cooler on wet ice and hand carried on a flight to the University of California, Davis for analysis. Upon receipt, 3 μL of whole blood was removed for Coulter counter analysis, 3 μL of whole blood was removed for blood smear analysis, and the remainder of the blood was used for flow cytometry.

2.3. Flow cytometry

Flow cytometry was used for initial ploidy assignment for both California and Idaho fish to which results from Coulter counter and blood smears were compared. Blood was prepared for flow cytometry following Schreier et al. (2013). Briefly, 25 μL of erythrocytes were collected from the bottom of the FACS tube and transferred to a new FACS tube containing 1 mL ACD. Samples were mixed and the concentration of erythrocytes was calculated for each sample using a hemocytometer. A final cell suspension concentration of ~1.0×10^6 cells/mL was created and 500 μL of the suspension were transferred to a new FACS tube and mixed with 1.0 mL Vindelov's propidium iodide solution (PI) and incubated on ice in the dark for 30 min. Vindelov's PI was created as follows: 121 mg Tris base (10 mM), 58.4 mg NaCl

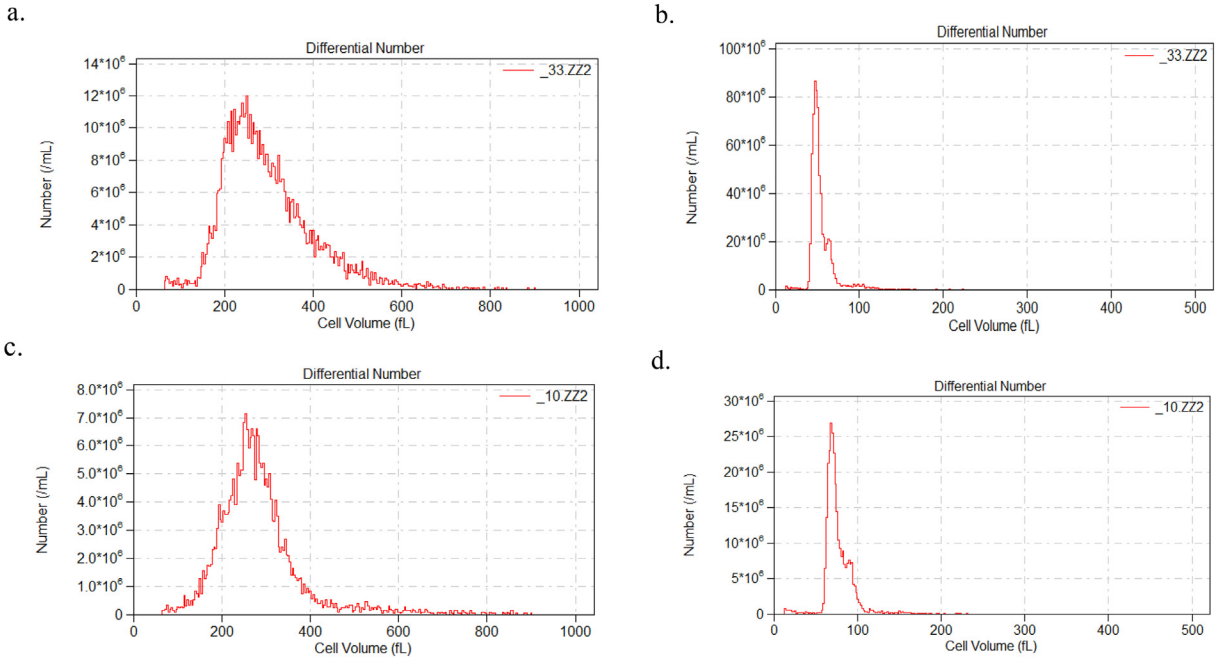

Fig. 2. Sample Coulter counter measurements of whole erythrocytes (a) and nuclei (b) from a single 8N individual, and whole erythrocytes (c) and nuclei (d) from a single 12N individual.

(10 mM), 1 mg RNase (DNase-free), 5.01 mg PI (7×10^{-5}M), 0.1 ml Nonidet P-40, Q.S. to 100 ml using distilled water to pH 8.0. Sample fluorescence was analyzed after the 30 min incubation period using a FACScan Analyzer (Becton Dickinson, Franklin Lakes, NJ, United States) at the University of California Davis Flow Cytometry Core Facility. Mean fluorescence was measured using FlowJo software v3.05260 (Becton Dickinson, Franklin Lakes, NJ, United States). Two drops of Trout Erythrocyte Nuclei (TEN; BioSure® Inc., Grass Valley, CA, USA) or 25 µl of triploid rainbow trout (*Oncorhynchus mykiss*) blood obtained from Nimbus Hatchery, Gold River, California, were prepared in parallel and included as an internal DNA content standard. The DNA content of each experimental sample was estimated by comparing its fluorescence to the fluorescence and genome size of the TEN/trout control by the equation DNA (pg) = 5.24 x (*S*/*T*), where *S* and *T* correspond to the mean fluorescence of the white sturgeon erythrocytes and TEN/trout erythrocytes, respectively. For samples analyzed with triploid rainbow trout blood, a genome size of 7.86 pg was used. Ten thousand cells were measured per sample.

2.4. Coulter counter

For Coulter counter analysis, the Z2 Coulter® Particle Count and Size Analyzer (Beckman Coulter, Inc., Brea, CA, United States) was used. Three µL of blood were deposited from syringes directly into two 25 mL cuvettes, one containing 10 mL of Isoton II Diluent and 3 drops of Zap-oglobin II lytic reagent for nuclei measurements (Beckman Coulter Inc., Brea CA) and one containing only Isoton II Diluent for whole erythrocyte analysis. The cuvettes were gently inverted several times to mix. Thirty samples of each type (nuclei and whole erythrocyte) were run in triplicate to verify consistency between runs, after which each sample was only run once. For the first California sampling event that included 8N and 10N individuals, Coulter counter samples were analyzed on Day 0 (day of collection), and then stored in a refrigerator (4 ± 1 °C). On day 3 and day 6, after one hour acclimation to room temperature, the samples were re-analyzed to evaluate the stability of samples over time. For the second California sampling event that included 8N and 12N individuals and all Idaho sampling events, samples were only analyzed once. For each sample, the aperture tube was placed into the cuvette and triplicate 500 µl samples drawn through the 100 µm diameter orifice using the Coulter counter. A mean of 20,000 cells were measured per sample. For each sample from the first California sampling event (8N and 10N samples), mean, median and mode of both the nuclei and whole erythrocytes were measured by the Coulter counter and the mode was recorded as the representative value for the nuclei and whole erythrocyte volume (μm^3) and diameter (µm) (see Results for justification). In subsequent analyses of California and Idaho 8N and 12N samples, only the mode value of the nuclear volume was recorded, as this was determined to be the most stable and accurate measurement (see Results for justification).

2.5. Blood smears

To create blood smears, 3 µL of blood were pipetted from the syringes onto a glass slide, and the blood was gently smeared across the surface of the slide using a second, clean glass slide held at a 45° angle. Two blood smears were created for each fish. After drying completely, blood smears were placed in a microscope slide tray and fixed in methanol for 2 min. They were then stained in Wright Giemsa (Sigma Aldrich®, St. Louis, Missouri; 80% methanol, 19% glycerin, 1% Giemsa's stain) for 30 s. The slides were then soaked for 5 min in deionized water, followed by twenty dips in fresh deionized water. After overnight drying, blood smears were imaged using a Lumenera® Corp Infinity 2 microscope camera using 40× magnification, with enough images taken per slide to obtain 40 intact cells for measurement. Images were analyzed using the software program Image J (available at: https://imagej.net/Downloads, date of last access: 9/2018), with calibration using a stage scale to approximately 6 pixels per micron. The longitudinal axis of 40 whole, mature erythrocyte cells per individual were measured for the Idaho fish, and both length and width of 40 cells per individual were measured for the California fish. Care was taken to avoid inclusion of leukocytes, which stain darker and are circular in shape, as these cells have different dimensions than erythrocytes. Thus, variable blood leukocyte levels between individuals could skew measurement values if leukocytes were included.

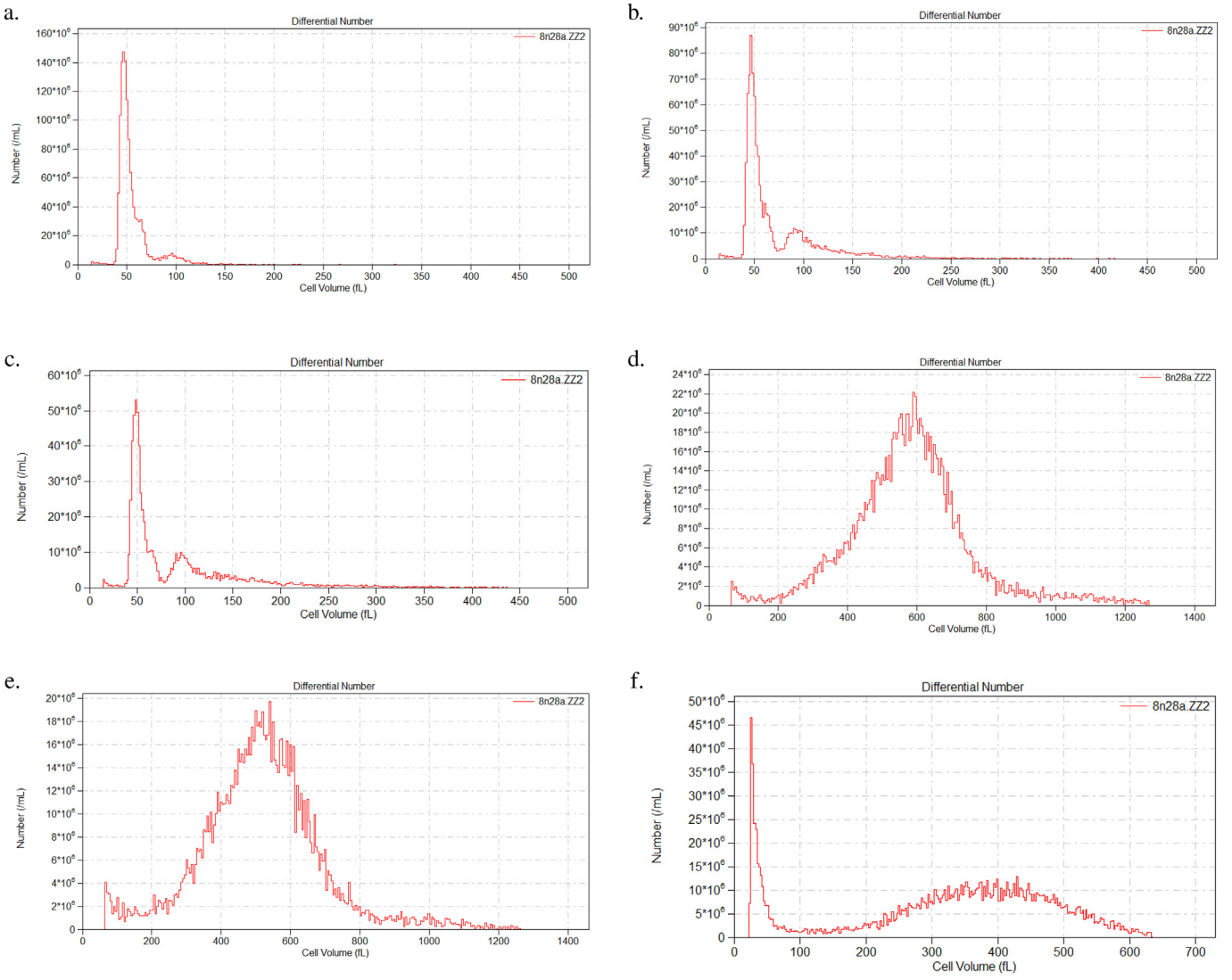

Fig. 3. Sample Coulter counter measurements from nuclei (a, b, c) and whole erythrocytes (d, e, f) at 0 days (a, d), 3 days (b, e), and 6 days (c, f) after initial sampling.

2.6. Data analysis

Data were analyzed in R (http://www.R-project.org). The distribution of modal nuclear volume (Coulter counter) and mean erythrocyte long-axis length (blood smears) for each ploidy class was tested for normality using a Shapiro-Wilk test. According to the non-parametric nature of the data, all Coulter counter data were analyzed using either a Mann-Whitney U or Kruskal-Wallis test followed by a post-hoc Dunn test. Mean erythrocyte length was calculated for each individual from blood smears and either a one-way ANOVA with post-hoc Tukey or Mann-Whitney U with post-hoc Dunn test was used to compare mean erythrocyte long-axis length between ploidy classes. Frequency histograms were created for mean erythrocyte long-axis length and nuclei volume to visually examine the distributions of this metric within and among ploidy classes. Linear discriminant analysis of mean erythrocyte long-axis length and width was conducted using the flipMultivariates package in R to further resolve ploidy classes using blood smear data.

3. Results

3.1. Flow cytometry

Flow cytometry for the California fish confirmed all putative 8N (n = 59), 10N (n = 29) and 12N (n = 30) individuals. Flow cytometry for the Idaho fish revealed 65 8N and 55 12N individuals, originating from five families (Table 1).

3.2. Coulter counter

Results from Shapiro-Wilk normality tests revealed that both the California and Idaho fish showed non-parametric distributions in the Coulter counter data (Table 2). A Kruskal-Wallis test revealed a significant effect of ploidy on erythrocyte nuclear volume in the California fish (Chi-squared = 101.95, df = 2, $p < .001$). A post-hoc Dunn's Multiple Comparisons test confirmed significant differences between all ploidy group comparisons ($p < .001$). A Mann-Whitney U test showed a significant effect of ploidy on erythrocyte nuclear volume in the Idaho fish (U = 3575, $p < .001$). Coulter counter analysis using the mode of nuclear volume to infer ploidy class showed 100% agreement with classification based on flow cytometry for all families from both the California and Idaho fish. Further, there was no overlap in the distribution of nuclear volumes for 8N, 10N and 12N individuals in either group of fish (Fig. 1. (c, d)). Nuclear volume was used rather than whole erythrocyte volume due to lower levels of variation in nuclear size (Fig. 2). Comparison of Coulter counter results on the day of sampling, three days after sampling and six days after sampling also revealed stability of nuclei measurements over time. In contrast, whole erythrocytes were not stable over time due to cell degradation and lysing;

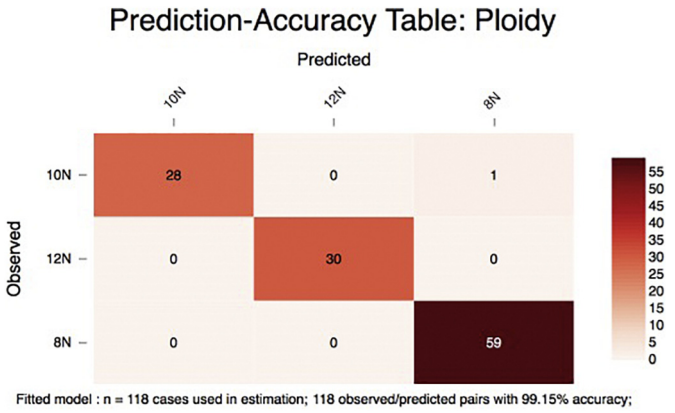

Fig. 4. Prediction accuracy table for 8N, 10N and 12N individuals based on whole erythrocyte length (LD1) and width (LD2). "Predicted ploidy" refers to the ploidy class predicted for a given individual based upon LD1 and LD2. "Observed ploidy" refers to the actual ploidy of that individual as determined by flow cytometry. Where both observed and predicted ploidies match, the linear discriminant analysis correctly predicted the ploidy of an individual based upon LD1 and LD2. Mismatch in observed and predicted ploidies indicates incorrect prediction. The colour saturation scale indicates the number of individuals within an observed/predicted category. n = 118 cases used in estimation; 118 observed/predicted pairs with 94.92% accuracy. LD1: $R^2 = 0.70$, LD2: $R^2 = 0.91$.

six days post sampling a distinct peak of nuclei developed and the peak for intact whole erythrocytes flattened and broadened (Fig. 3). The mode statistic was used rather than the mean in order to avoid skewing the data by inclusion of both small and large particles in the sample that were not nuclei (8N: SD of mean = ± 2.6, SD of mode = ± 0.2; 10N: SD of mean = ± 14.1, SD of mode = ± 1.2).

3.3. Blood smears

Shapiro-Wilk normality tests revealed normally distributed data in the California fish, and non-parametric data in the Idaho fish (Table 2). A one-way ANOVA followed by a post-hoc Tukey test revealed significant differences in erythrocyte long-axis lengths between all three ploidy classes in the California fish (one-way ANOVA, df = 2, F = 464.2, $p < .001$; post-hoc Tukey = $p < .001$ for all comparisons; 8N mean = 14.1 cm, 10N mean = 15.5 cm, 12N mean = 17.6 cm). Comparison between ploidy classes in the Idaho fish also showed significant differences between 8N and 12N ploidy classes (Mann-Whitney U, U = 3286, $p < .001$; 8N mean = 17.4 cm, 12N mean = 20.0 cm). The California population showed high variance in erythrocyte long-axis length within ploidy class and also exhibited overlap between 10N and both 8N and 12N classes, however, there was no overlap between 8N and 12N ploidy classes. In the California fish, we found that 38% of 8N and 10N individuals fell within an area of the distribution that contained both 8N and 10N fish, making these individuals indistinguishable from one another using blood smears alone (Fig. 1). The Idaho population also showed high variance within ploidy class as well as overlap between the 8N and 12N distributions (Fig. 1). In the Idaho fish, 71% of individuals fell within an area of the distribution containing both 8N and 12N fish. To improve classification accuracy, a linear discriminant analysis (LDA) incorporating both long-axis length and width measurements of whole erythrocytes was used. LDA had a prediction accuracy of 97% for 8N, 86% for 10N and 100% for 12N individuals, for an overall prediction accuracy of approximately 95%, based only on the California fish (Fig. 4).

4. Discussion

Our results confirm the accuracy and reliability of the Coulter counter for use in determining the ploidy of white sturgeon. The Coulter counter showed 100% agreement with ploidies assigned by flow cytometry, supporting an earlier white sturgeon study using a previous model of Coulter counter instrumentation with a channelyzer (Van Eenennaam et al., 1996). Our results also agree with those of an earlier study examining sunshine bass (female white bass *Morone chrysops* x male striped bass *Morone saxatilis*) that also found agreement between Coulter counter and flow cytometry results in distinguishing between ploidies (Harrell et al., 1998). Although not all studies testing the use of Coulter counter to determine ploidy found 100% accuracy, in one study the authors speculated that cell lysing and variable sample holding time were responsible for inaccuracies (Johnson et al., 1984). We recommend using the nuclear volume for ploidy determination using Coulter counter due to the stability of the nucleus in comparison to whole erythrocytes, which are prone to lysing after 3 days. Wattendorf (1986) reported that blood lysed in electrolyte could be stored in a refrigerator up to 36 h, and then warmed to room temperature prior to analysis of nuclei, and this study determined that comparable results could be obtained with lysed samples stored up to 6 days. We further recommend using the mode statistic rather than the mean or median, both of which are prone to being skewed by the presence of outlier particles that are not whole cells or nuclei, but are incidentally

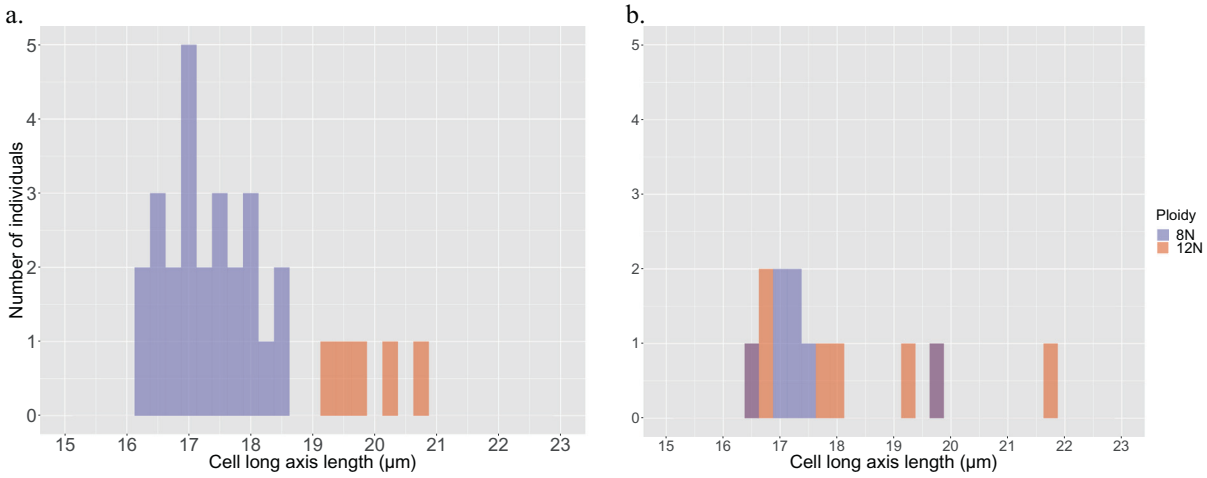

Fig. 5. Distribution of mean erythrocyte long-axis lengths for 8N (purple) and 12N (orange) individuals from (a) family 3 ($n = 30$) and (b) family 5 ($n = 15$) from the Idaho population measured using blood smears. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

measured by the Coulter counter.

The use of Coulter counter offers several advantages over flow cytometry, including lower instrumentation and per sample cost, ease of use and limited labor time. The Z2 Coulter® Particle Count and Size Analyzer cost is approximately $19,000 (including software), and per sample cost (not including labor) is ~$ 0.50. In contrast, a small benchtop laser flow cytometer costs ~$15,000–20,000 and per sample cost (not including labor) is ~ $3 per sample. Further, the Coulter counter is relatively easy to operate, whereas the flow cytometer requires more training to operate effectively. Approximately 10 samples per hour can be analyzed using the FACScan Analyzer (including sample preparation time), while Coulter counter allows for the analysis of approximately 60 samples per hour. Several chemicals used for flow cytometry, including the commonly used chemical propidium iodide, are toxic or carcinogenic. Cells must also be allowed to settle overnight prior to analysis, neither of which are required for Coulter counter analysis or blood smears. The Coulter counter can also provide near immediate results, allowing for sorting of fish by ploidy as they are sampled. Lastly, the Coulter counter can provide accurate results up to 6 days post sampling when samples are kept at 4 °C, whereas flow cytometry samples of fresh blood must be analyzed within 24–48 h of collection. The extended window of time for analysis using Coulter counter can allow hatcheries to send samples to other facilities for processing.

Although the least expensive method of ploidy analysis, blood smears varied in their ability to distinguish between ploidy classes. Our results revealed statistically significant differences in erythrocyte long-axis lengths between all three ploidy classes in both populations, agreeing with previous studies of a *C. idella* x *H. nobilis* hybrid and turbot (*Scophthalmus maximus*) that both showed significant differences in erythrocyte length between ploidies using blood smears (Beck and Biggers, 1983; Cal et al., 2005). Despite these statistically significant differences in erythrocyte length, we observed a large degree of overlap in the 8N/10N distributions in the California fish and the 8N/12N distributions in the Idaho fish as well as variability between both families and populations, making accurate ploidy assignment difficult using blood smears (Fig. 5). Although other studies demonstrate 100% accuracy of ploidy assignment using longitudinal long-axis length and width of blood smears in a variety of fish species including shortnose sturgeon, (e.g. Espinosa et al., 2005; Benfey et al., 1984; Beyea et al., 2005), our results show that this technique is not 100% accurate in white sturgeon. However, it is important to note that the study examining shortnose sturgeon only examined 8N and 12N individuals and did not look at fish with intermediate ploidy (10N), many of which we found to be indistinguishable using blood smears (Beyea et al., 2005). Although we did not analyze any 10N Idaho fish, these individuals would be indistinguishable from the 8N and 12N Idaho individuals given the overlap of the 8N/12N distributions in this population.

There are however some limited instances in which blood smears can be used for ploidy determination. For the California fish, there was no overlap in the distribution of 8N and 12N individuals, and thus this tool could be used by caviar farms that are mainly concerned with excluding 12N individuals from their broodstock to prevent creating 10N progeny. Although blood smears offer the lowest instrumentation and materials cost of the methods tested here, it is important to note the significant time investment in production and analysis, which can take anywhere between 20 and 30 min per individual including the production, staining, imaging and analysis of smears. While LDA using both length and width of whole erythrocytes improved overall prediction accuracy (only 7% of 8N and 10N individuals were indistinguishable from one another versus 38% using only the long-axis length), it also significantly increased analysis time for blood smears. For this reason, blood smears may be most useful for caviar farms aiming to identify 12N individuals in a small number of broodstock rather than conservation hatcheries looking to screen many individuals before release into the wild. Finally, when using blood smears in a new species or population, it is essential to first validate the accuracy of this technique by comparison with flow cytometry in a subset of individuals from multiple families to examine potential overlap in erythrocyte long-axis distributions, as we noted a high degree of variation in erythrocyte long-axis length between populations as well as variation between families within a single population (Fig. 5). We recommend the validation of at least two families per ploidy group of interest (within each population) before using blood smears for ploidy determination. Depending on the degree of overlap observed between the two families, additional families may be needed to fully characterize overlap between ploidies. To that end, we also recommend further validation of blood smears from 12N individuals in the California white sturgeon population examined in this study before using blood smears to ensure no overlap with the 8N distribution. We currently cannot recommend the use of blood smears in the Idaho populations of white sturgeon due to high variance in cell size. Further, based on our results we do not recommend the use of blood smears to determine ploidy in white sturgeon unless a population has been validated and ploidy distributions fully characterized.

5. Conclusion

Our data show that determining the mode of erythrocyte nuclear volume using a Coulter counter is equal to flow cytometry in terms of its accuracy of ploidy assignment in white sturgeon. Further, Coulter counter provides a simple and efficient technique for determining ploidy, and samples can be analyzed up to 6 days after collection. Although the Coulter counter instrument can be prohibitively expensive for some small aquaculture facilities, it is recommended for any sturgeon conservation hatchery that needs to screen both broodstock and incidence of 12N's in families to be released. Partnerships between universities with access to a Coulter counter and hatcheries could also facilitate the use of Coulter counters in sturgeon aquaculture for those facilities unable to purchase one. Despite several important considerations and exceptions in the use of blood smears to determine ploidy in white sturgeon, we conclude that blood smears can be useful for identifying 12N individuals in small aquaculture facilities and in some populations after sufficient validation using an alternative method such as Coulter counter or flow cytometry.

Declarations of interest

None.

Funding

This study was funded by the Western Regional Aquaculture Center from the USDA National Institute of Food and Agriculture [grant numbers 2014-38500- 22309, 2014-38500-2230]. Funding sources had no involvement in study design, data collection, analysis or article preparation.

Acknowledgements

The authors would like to thank the Kootenai Tribe of Idaho for sampling and supplying blood from the Idaho population, as well as for preparing blood smears from the Idaho fish. The authors would also like to thank Michaiah Leal, Alyssa Benjamin, and Shannon Kieran from the University of California, Davis for assistance in blood collection and flow cytometry analysis.

References

Allen, S.K.J., 1983. Flow cytometry: assaying experimental polyploid fish and shellfish. Aquaculture 33, 317–328. https://doi.org/10.1016/0044-8486(83)90412-X.

Beck, M.L., Biggers, C.J., 1983. Erythrocyte measurements of diploid and triploid Ctenopharyngodon idella X Hypophthalmichthys nobilis hybrids. J. Fish Biol. 22,

497–502. https://doi.org/10.1111/j.1095-8649.1983.tb04770.x.
Benfey, T.J., Sutterlin, A.M., Thompson, R.J., 1984. Use of erythrocyte measurements to identify triploid salmonids. Can. J. Fish. Aquat. Sci. 41, 980–984. https://doi.org/10.1139/f84-112.
Beyea, M.M., Benfey, T.J., Kieffer, J.D., 2005. Hematology and stress physiology of juvenile diploid and triploid shortnose sturgeon (Acipenser brevirostrum). Fish Physiol. Biochem. 31, 303–313. https://doi.org/10.1007/s10695-005-1552-y.
Birstein, V.J., 2005. Phylogeny and evolution of Acipenseriformes: new molecular and genetic data create new puzzles. In: Gall, Y.M., Kolchinsky, E.I. (Eds.), Evolutionary Biology: History and Theory. Saint-Petersburg Institute of History Publishers, St. Petersburg, pp. 231–269.
Birstein, V.J., Hanner, R., DeSalle, R., 1997. Phylogeny of the Acipenseriformes: cytogenetic and molecular approaches. Environ. Biol. Fish 48 (1), 157–163. https://doi.org/10.1023/A:1007366100353.
Blacklidge, K.H., Bidwell, C.A., 1993. Three ploidy levels indicated by genome quantification in Acipenseriformes of North America. J. Hered. 84 (6), 427–430.
Cal, R.M., Vidal, S., Camacho, T., et al., 2005. Effect of triploidy on turbot haematology. Comp. Biochem. Physiol. A Physiol. 141, 35–41. https://doi.org/10.1016/j.cbpb.2005.03.007.
Cogswell, A.T., Benfey, T.J., Sutterlin, A.M., 2002. The hematology of diploid and triploid transgenic Atlantic salmon (Salmo salar). Fish Physiol. Biochem. 24, 271–277. https://doi.org/10.1023/A:1015020716456.
Duke, S., Anders, P., Ennis, G., et al., 1999. Recovery plan for Kootenai River white sturgeon (Acipenser transmontanus). J. Appl. Ichthyol. 15, 157–163.
Espinosa, E., Josa, A., Gil, L., et al., 2005. Triploidy in rainbow trout determined by computer-assisted analysis. J. Exp. Zool. 303, 1007–1012. https://onlinelibrary.wiley.com/doi/abs/10.1002/jez.a.223.
Garcia-Abiado, M.R., Dabrowski, K., Christensen, J.E., et al., 1999. Use of erythrocyte measurements to identify triploid saugeyes. N. Am. J. Aquac. 61, 319–325.
Harrell, Reginal M., Van Heukelem, William, Kerby, J. Howard, 1998. A comparison of triploid induction validation techniques. Progress. Fish Cult. 60, 221–226. https://doi.org/10.1577/1548-8640(1998)060<0221:ACOTIV>2.0.CO;2.
Havelka, M., Hulak, M., Rab, P., et al., 2014. Fertility of a spontaneous hexaploid male Siberian sturgeon, *Acipenser baerii*. BMC Genet. 15(5). https://doi.org/10.1186/1471-2156-15-5.
Havelka, M., Bytyutskyy, D., Symonova, R., et al., 2016. The second highest chromosome count among vertebrates is observed in cultured sturgeon and is associated with genome plasticity. Genet. Sel. Evol. 48 (12). https://doi.org/10.1186/s12711-016-0194-0.
Hedrick, R.P., McDowell, T.S., Rosemark, R., et al., 1991. Two Cell Lines from White Sturgeon. 120. Transactions of the American Fisheries Society, pp. 528–534. https://doi.org/10.1577/1548-8659(1991)120<0528:TCLFWS>2.3.CO;2.
Hildebrand, L.R., Schreier, A. Drauch, Lepla, K., et al., 2016. Status of White Sturgeon (Acipenser transmontanus) throughout the species range, threats to survival, and prognosis for the future. J. Appl. Ichthyol. 32 (Suppl. 1), 261–312. https://doi.org/10.1111/jai.13243.
Johnson, O.W., Rabinovich, P.R., Utter, F.M., 1984. Comparison of the reliability of a coulter counter with a flow cytometer in determining ploidy levels in Pacific salmon. Aquaculture 43, 99–103. https://doi.org/10.1016/0044-8486(84)90014-0.
Lemoine, H.L., Smith, L.T., 1980. Polyploidy induced in brook trout by cold shock. Trans. Am. Fish. Soc. 109, 626–631. https://doi.org/10.1577/1548-8659(1980)109<626:PIIBTB>2.0.CO;2.
Schreier, A.D., Van Eenennaam, J.P., 2018. 2017 Genetic diversity monitoring of white sturgeon in the Kootenai Tribe of Idaho native fish conservation aquaculture program and study of 10N reproductive development. University of California, Davis, pp. 17.
Schreier, A.D., Gille, D., Mahardja, B., et al., 2011. Neutral markets confirm the octoploid origin and reveal spontaneous autopolyploidy in white sturgeon Acipenser transmontanus. J. Appl. Ichthyol. 27, 24–33. https://doi.org/10.1111/j.1439-0426.2011.01873.x.
Schreier, A.D., May, B., Gille, D.A., 2013. Incidence of spontaneous autopolyploidy in cultured populations of white sturgeon, *Acipenser transmontanus*. Aquaculture 416–417, 141–145. https://doi.org/10.1016/j.aquaculture.2013.09.012.
Sezaki, K., Kobayashi, H., Nakamura, M., 1977. Size of erythrocytes in the diploid and triploid specimens of Carassius auratus langsdorfi. Jpn. J. Ichthyol. 24, 135–140.
Thorgaard, G.H., Rabinovitch, P.S., Shen, M.W., et al., 1982. Triploid rainbow trout identified by flow cytometry. Aquaculture 29 (3–4), 305–309. https://doi.org/10.1016/0044-8486(82)90144-2.
Valenti, R.J., 1975. Induced polyploidy in Tilapia aurea (Steindachner) by means of temperature shock. J. Fish Biol. 7, 519–528. https://doi.org/10.1111/j.1095-8649.1975.tb04627.x.
Van Eenennaam, A.L., Van Eenennaam, J.P., Medrano, J.F., et al., 1996. Rapid verification of meiotic gynogenesis and ploypoidy in white sturgeon (Acipenser transmontanus Richardson). Aquaculture 147, 177–189. https://doi.org/10.1016/S0044-8486(96)01369-5.
Van Eenennaam, J.P., Chapman, F.A., Jarvis, P.L., 2004. Sturgeons and paddlefish of North America. In: LeBreton, G.T.O., Beamish, F.W.H., McKinley, R.S. (Eds.), Aquaculture. Springer, Netherlands: Dordrecht, pp. 277–311.
Wattendorf, R.J., 1986. Rapid identification of triploid grass carp with a coulter counter and channelyzer. Progress. Fish Cult. 48, 125–132. https://doi.org/10.1577/1548-8640(1986)48<125:RIOTGC>2.0.CO;2.
Young, S., Lewandowski, C., Ponce, J., et al., 2016. In: Associates, D.J.W.A. (Ed.), Kootenai River White Sturgeon and Burbot Aquaculture Techniques Manual. Kootenai Tribe of Idaho.